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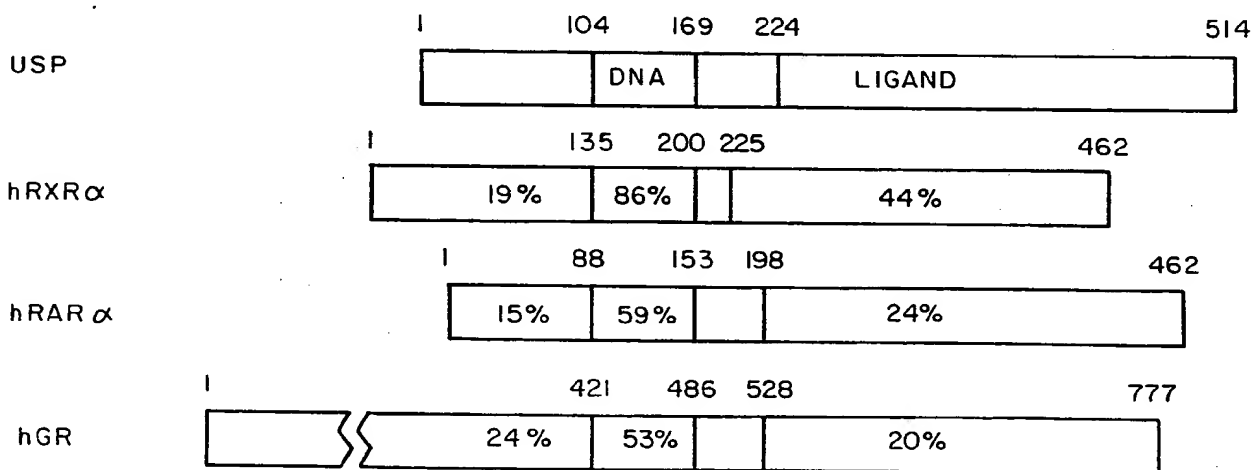
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(54) Title: INSECT RETINOID RECEPTOR COMPOSITIONS AND METHODS



(57) Abstract

The present invention relates to the discovery of novel insect receptor polypeptides, which modulate transcription of certain genes by binding to cognate response elements. The novel receptors of the invention are activated when the cells are exposed to retinoic acid. The invention provides DNAs encoding the novel receptors; expression vectors; cells transformed with such expression vectors; cells co-transformed with such expression vectors and with test vectors to monitor activation of the receptors to modulate transcription; and methods of using such co-transformed cells in screening for compounds which are capable of activation of the receptors and for compounds capable of interfering with such activation as potential potent insecticides. The invention also provides DNA and RNA probes for identifying DNA's encoding retinoid receptors of insects and other animals as shown in the figure, to which the novel receptors of the invention belong.

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# INSECT RETINOID RECEPTOR COMPOSITIONS AND METHODS

## TECHNICAL FIELD

The present invention concerns novel, steroid-hormone-receptor-like proteins and methods of making and using same.

5 More particularly, the invention relates to such proteins that occur in insects and that have transcription-modulating effects, at affected promoters, that are responsive to the presence of retinoic acid and other retinoids and vitamin A metabolites in the media  
10 bathing insect cells that harbor the proteins.

## BACKGROUND OF THE INVENTION

The retinoids comprise a group of compounds including retinoic acid, retinol (vitamin A), retinal,  
15 retinyl acetate, retinyl palmitate, and a series of natural and synthetic derivatives of these compounds, that together exert profound effects on development and differentiation in a wide variety of systems. Although early studies focused on the effects of retinoids on  
20 growth and differentiation of epithelial cells, their actions have been shown to be widespread. Many recent studies have examined the effects of these molecules on a variety of cultured neoplastic cell types, including the human promyelocytic leukemia cell line, HL60, where  
25 retinoic acid appear to be a potent inducer of granulocyte differentiation. In F9 embryonal carcinoma cells, retinoic acid will induce the differentiation of parietal endoderm, characteristic of a late mouse blastocyst. Retinoic acid also appears to play an  
30 important role in defining spatio-temporal axes in the developing avian limb and the regenerating amphibian limb.

R tinoic acid has been shown t induce th transcription of s veral cDNAs whose g ne products have  
35 been isolated by diff r ntial screening, supp rting the

hypothesis that retinoic acid exerts its action via modulation of gene expression, mediated by a receptor protein, in a fashion analogous to the means by which steroid and thyroid hormones influence their target genes.

The ability to identify compounds able to affect transcription of insect genes could be of significant value in identifying compounds useful as insecticides. Of significance in this regard would be the identification of receptor proteins which modulate transcription of insect genes.

Systems useful for monitoring solutions, body fluids and the like for the presence of retinoic acid, vitamin A or metabolites of the latter would be of value in various analytical biochemical applications and, potentially, medical diagnosis.

Through molecular cloning studies it has been possible to demonstrate that mammalian receptors for steroid, retinoid and thyroid hormones are all structurally related and comprise a superfamily of regulatory proteins that are capable of modulating specific gene expression in response to hormone stimulation by binding directly to cis-acting elements (Evans, Science 240, 889 (1988); Green and Chambon, Trends genet. 4, 309 (1988)). Structural comparisons and functional studies with mutant receptors have made it possible to discern that these molecules are composed of a series of discrete functional domains, most notably, a DNA-binding domain that is composed typically of 66 - 68 amino acids, including two zinc fingers, and an associated carboxy terminal stretch of approximately 250 amino acids which comprises the ligand-binding domain (reviewd in Evans, supra).

An important advance in the characterization of this family has been the delineation of a growing list of gene products isolated by low-stringency hybridization

techniques which possess the structural features of hormone receptors. A retinoic acid dependent transcription factor, referred to as RAR-alpha (retinoic acid receptor-alpha), has been identified. Subsequently, two additional RAR-related genes have been isolated, and there are now at least three different RAR subtypes (alpha, beta and gamma) known to exist in mice and humans. These retinoic acid receptors (RARs) share homology to the superfamily of steroid hormone and thyroid hormone receptors and have been shown to regulate specific gene expression by a similar, ligand-dependent mechanism (Umesono et al., Nature 336, 262 (1988)). These RAR subtypes are expressed in distinct patterns throughout development and in the mature organism.

Recently, another retinoic acid-dependent transcription factor, designated RXR-alpha, has been identified in cDNA libraries prepared from human cells. RXR-alpha differs significantly in primary amino acid sequence from human RAR-alpha and other known members of the mammalian steroid/ thyroid hormone superfamily of receptors. RXR-alpha is activated to effect trans-acting transcription activation ("trans-activation") in mammalian and insect cells exposed to retinoic acid and retinal and mammalian cells exposed to a number of synthetic super-retinoids. The dose-response of trans-activation by RXR-alpha in CV-1 monkey kidney cells exposed to retinoic acid differs significantly from that by human RAR-alpha. See commonly owned, co-pending United States Patent Application Serial No. 07/478,071, filed February 9, 1990, which is incorporated herein by reference.

Other information helpful in the understanding and practice of the present invention can be found in commonly assigned, co-pending United States Patent Application Serial Nos. 108,471, filed October 20, 1987; 276,536, filed November 30, 1988; 325,240, filed March

17, 1989; 370,407, filed June 22, 1989; and 438,757, filed November 16, 1989, all of which are incorporated herein by reference.

As will be detailed further below, the  
5 receptors of the invention modulate transcription of genes by binding to thyroid hormone response elements positioned operatively, with respect to the promoters of the genes, for such modulation to occur upon the binding of the receptor. Among such thyroid hormone response  
10 elements are TREp, the beta-retinoic acid response element and closely related elements (see Application Serial No. 438,757, filed November 16, 1989), and the estrogen response element (see Application Serial No. 325,240, filed March 17, 1989).

15

#### SUMMARY OF THE INVENTION

We have discovered in insect cells novel receptors which are activated to modulate transcription of certain genes in such cells, when the cells are  
20 exposed to a retinoid, such as retinoic acid, retinol, retinal, retinyl acetate, or retinyl palmitate. The novel insect receptors differ significantly from the known RAR-alpha, beta and gamma receptors in primary sequence but share significant homology with RXR-alpha.

25 One of the receptors of the invention, XR2C, from *Drosophila melanogaster*, has been mapped to the *Drosophila* ultraspiracle locus, a locus known to be required both maternally and zygotically for pattern formation. Thus, compounds which interfere with, or  
30 otherwise alter, trans-activation by the receptors of the invention would be expected to be insecticidal.

The invention provides DNAs encoding the novel insect receptors, including expression vectors for  
35 expression of the receptors in animal cells, especially insect cells, transformed with such expression vectors, cells co-transformed with such expression vectors and

with test vectors to monitor activation of the receptors to modulate transcription, when the cells are exposed to a compound which leads to such activation, and methods of using such co-transformed cells in (1) assaying fluids exposed to the cells for the presence of compounds (e.g., retinoic acid) capable of activating the receptors for transactivation, (2) screening for compounds which are capable of leading to such activation of the receptors, and (3) screening for compounds which are antagonists of transactivation by the receptors (i.e., compounds capable of blocking trans-activation by the receptors in cells exposed to both the blocking compound and a compound (e.g., retinoic acid) that would normally activate the receptors to trans-activation). Such antagonists of transactivation are likely to be toxic or lethal to insects.

The invention also provides DNA and RNA probes for identifying DNA's encoding RXR's, and particularly insect RXR's (i.e., receptors of the same class as human RXR-alpha, including XR2C of the present invention).

The invention also provides a method for making the receptors of the invention by expressing in bacteria DNAs, which encode the receptors. These bacterially produced receptors are useful for assessing the ability of receptor agonists or antagonists to bind to the receptor.

Animal cells, and especially insect cells, according to the invention, in which receptors are expressed from DNAs of the invention, can be employed, as more fully taught in the examples, in assaying fluids for the presence of retinoic acid.

As indicated above, animal cells, and especially insect cells, of the invention can also be employed to screen compounds of potential value as insecticides.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the coding sequence of a DNA segment which comprises a segment encoding XR2C, a receptor polypeptide according to the invention. The

5 Figure also shows the amino acid sequence of XR2C. In said amino acid sequence, the 66-amino acid DNA binding domain is amino acids 104 - 169. The DNA segment, whose sequence is shown in Figure 1, is the segment (but for the EcoRI site overhangs) inserted at the EcoRI site of  
10 pBluescript<sup>®</sup> phagemid SK(+) to make pXR2C8, a DNA of the invention.

Figure 2 shows the extent of amino acid identity (i.e., "homology") between the DNA binding domain ("DNA") and ligand binding domain ("LIGAND") of  
15 XR2C (designated in the Figure as "USP" because it is the product of the ultraspiracle locus of *D. melanogaster*) (taken to have 100 % identity in both domains) and the corresponding domains of human RXR-alpha, human retinoic acid receptor-alpha (hRAR-alpha), and human  
20 glucocorticoid receptor (hGR). The numbers outside the boxes in the Figure are the numbers, in the primary sequences of the proteins, of the amino acids defining the two domains and the amino- and carboxy-termini.

## 25 DETAILED DESCRIPTION OF THE INVENTION

The invention concerns novel polypeptides, which (1) in an insect cell in culture, the medium of which comprises retinoic acid at a concentration greater than about  $5 \times 10^{-7}$  M, increase the rate of transcription  
30 from a promoter linked to TREp operatively for activation of transcription by hRXR-alpha; and (2) has a DNA binding domain of about 66 (i.e., 64 - 68) amino acids with 10 Cys residues, more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha and  
35 less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR.



Thus, the invention entails a double-stranded DNA which comprises a segment, which consists of a continuous sequence of double-stranded, amino-acid-encoding triplets including, at its 5'-end, a triplet  
5 encoding a translational start codon, and, at its 3'-end, a triplet encoding a translational stop codon, said continuous sequence encoding a polypeptide which: (1) in an insect cell in culture, the medium of which comprises retinoic acid at a concentration greater than about  $5 \times 10^{-7}$  M, increases the rate of transcription from a  
10 promoter linked to TREp operatively for activation of transcription by hRXR-alpha; and (2) has a DNA binding domain of about 66 amino acids with 10 Cys residues, more than about 75 % amino acid identity in comparison with  
15 the DNA binding domain of hRXR-alpha and less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR.

Further, the invention encompasses a DNA according to the invention which is an expression vector  
20 which is operative in an animal cell (preferably an insect cell) in culture to make the protein encoded by the continuous sequence of amino-acid-encoding triplets in the DNA by expression of said continuous sequence in said cell.

25 Further, the invention entails an animal cell (preferably an insect cell) in culture which is transformed with an expression vector, which is operative in the cell to make a polypeptide, by expression of a DNA segment, which consists of (a) a continuous sequence of  
30 double-stranded, amino acid-encoding triplets including, at the 5'-end, a triplet encoding a translational start codon, the sequence of amino acids encoded by said continuous sequence of triplets being the primary sequence of the polypeptide, and (b) at the 3'-end, a  
35 triplet encoding a translational stop codon, the polypeptide: (1) in an insect cell, in a culture, the

medium of which comprises retinoic acid at a concentration greater than about  $5 \times 10^{-7}$  M, increasing the rate of transcription from a promoter linked to TREp operatively for activation of transcription by hRXR-alpha; and (2) having a DNA binding domain of about 66 amino acids with 10 Cys residues, more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha and less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR.

Among the animal cells of the invention are those which are co-transformed with a test vector which comprises (a) a promoter that is operable in the cell, (b) a thyroid hormone response element, and (c) a DNA segment encoding a reporter protein, said reporter-protein-encoding DNA segment linked to said promoter operatively for transcription therefrom and said thyroid hormone response element linked to said promoter operatively for increasing transcription therefrom upon binding at said response element of the protein made by expression from the expression vector, said cell being such that the rate of production of said reporter protein in a culture of said cells, in the medium of which retinoic acid is present at  $5 \times 10^{-7}$  M, is significantly increased (i.e., increased by more than the experimental error in the measurement) over said level of production in a culture of the cells, in the medium of which retinoic acid is present at  $10^{-8}$  M.

The invention also entails a method of testing a compound for ability to activate the transcription-activating effects of a polypeptide, which (1) in an insect cell in culture, the medium of which comprises retinoic acid at a concentration greater than about  $5 \times 10^{-7}$  M, increases the rate of transcription from a promoter linked to TREp operatively for activation of transcription by hRXR-alpha; and (2) has a DNA binding

domain of about 66 amino acids with 10 Cys residues, more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha and less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR, which method comprises: (A) adding, to a first concentration, said compound to the culture medium of a first culture of animal (preferably insect) cells, which are co-transformed with (i) an expression vector, which is operative in the cells to make said polypeptide and (ii) a test vector, which comprises (a) a promoter that is operable in the cells, (b) a thyroid hormone response element, and (c) a DNA segment encoding an reporter protein, said reporter-protein-encoding DNA segment linked to said promoter operatively for transcription therefrom and said response element linked to said promoter operatively for increasing transcription therefrom upon binding at the element of the polypeptide made by expression from the expression vector, said cells being such that the rate of production of said reporter protein in a culture of the cells, in the medium of which retinoic acid is present at  $5 \times 10^{-7}$  M, is significantly increased over said level of production in a culture of the cells, in the medium of which retinoic acid is present at  $10^{-8}$  M, and (B) comparing the rate of production of said reporter protein after addition of the compound to said first culture to said first concentration with the rate of production of said reporter protein in a control culture of said cells, to which the compound is added to a second concentration that is significantly (i.e., measurably) different from said first concentration. Preferably, in this method of the invention, said first culture and said control culture are two subcultures of a common culture and said second concentration is 0.

35           The invention entails also a method of testing a first compound for ability to affect trans-activation,

by a receptor of the invention as activated by a second compound, known to activate trans-activation by the receptor. This method is carried out using at least two (i.e., a first and a second) culture of an animal

5 (preferably insect) cell of the invention, which is co-transformed with an expression vector, from which the receptor of the invention is made, and a test vector, as described supra wherefrom an indicator protein (i.e., a reporter protein) is made at a rate dependent on trans-

10 activation through binding of activated receptor to a thyroid hormone response element. In the method, the ratio of the concentration of the first compound to that of the second compound differs in each of the cultures. The affect of the first compound on transactivation by

15 the receptor activated by the second compound is assessed by comparing the rate of production of the reporter protein in the various cultures. The preferred second compound is retinoic acid, but other retinoids or any compound known (e.g, from the test method of the

20 invention described in the immediately preceding paragraph) to activate trans-activation by the receptor of the invention can be employed as second compound. First compounds identified in this testing method of the invention as affecting trans-activation, and especially

25 those that block or antagonize activation of trans-activation, are likely to be useful as insecticides. This method of testing of the invention can also be defined as follows: A method of testing a first compound for ability to affect the activation by a second compound

30 of the transcription-activating effects of a receptor polypeptide, which (1) in an insect cell in culture, the medium of which comprises retinoic acid at a concentration greater than about  $5 \times 10^{-7}$  M, increases the rate of transcription from a promoter linked to TREp

35 peratively for activation of transcription by hRXR-alpha; and (2) has a DNA binding domain of about 66 amino

acids with 10 Cys residues, more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha and less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR, said method comprising (A) adding said first and second compounds, at a first ratio of concentrations, to a first culture of animal cells and adding said first and second compounds, at a second ratio of concentrations, significantly different from said first ratio, to a second culture of animal cells, said animal cells in both of said cultures being substantially the same and being co-transformed with (i) an expression vector, which is operative in the cells to make said receptor polypeptide, and (ii) a test vector, which comprises (a) a promoter that is operable in the cells, (b) a thyroid hormone response element, and (c) a DNA segment encoding a reporter protein, said reporter-protein-encoding DNA segment linked to said promoter operatively for transcription therefrom and said response element linked to said promoter operatively for increasing transcription therefrom upon binding at the element of said receptor polypeptide, said cells being such that the rate of production of said reporter protein in a culture of the cells, in the medium of which retinoic acid is present at  $5 \times 10^{-7}$  M, is significantly increased over said level of production in a culture of the cells, in the medium of which retinoic acid is present at  $10^{-8}$  M, and (B) comparing the rate of production of said reporter protein in said first culture with the rate of production of said reporter protein in said second culture, provided that the concentrations of said second compound in both cultures are such that, if said first compound were not present in said cultures, the concentrations of second compound would be sufficient to activate transactivation by said receptor protein in said cells. Preferably, the various cultures, in which different concentration ratios

of first and second compound are compared in accordance with the method for affect on rate of production of reporter protein, are all subcultures of a common culture, all have the same concentration of second  
5 compound and, in one of these cultures in a set in which different concentration ratios are being assessed, the concentration of first compound is 0.

Futher, the invention encompasses various probes, which can be used to identify genes for receptors  
10 related to those with which the present invention is concerned. In this regard, particular reference is made to Example IV below. More particularly, the invention entails a DNA or RNA which is labelled for detection and comprises a segment of at least 20 bases in length which  
15 has the same sequence as (i) a segment of the same length from the DNA segment from bases 1 - 2271, inclusive, of the DNA illustrated in Figure 1 or (ii) the complement of said segment.

The invention also encompasses a method of  
20 making a polypeptide which: (1) in an insect cell in culture, the medium of which comprises retinoic acid at a concentration greater than about  $5 \times 10^{-7}$  M, increases the rate of transcription from a promoter linked to TREP operatively for activation of transcription by hRXR-  
25 alpha; and (2) has a DNA binding domain of about 66 amino acids with 10 Cys residues, more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha and less than about 60 % amino acid identity in comparison with the DNA binding domain of  
30 hGR, which method comprises culturing bacterial cells which are transformed with an expression vector operable in said cells to express a DNA which comprises a segment, which consists of a continuous sequenc of d uble-stranded, amin -acid-enc ding triplets including, at its  
35 5'-end, a tripl t encoding a translational start codon, and, at its 3'-end, a triplet encoding a translational

stop codon, said continuous sequence encoding said polypeptide. In this method of the invention, *E. coli* is the preferred bacterial species. Any of a number of bacterial expression vectors are well known to those skilled in the art that could be employed in the method of the invention. Among these are the prokaryotic expression vectors pNH8A, pNH16A and pNH18A available from Stratagene, La Jolla, California USA.

Further information on the invention is provided in the following examples and description of a deposit.

#### Example I

The KpnI/SacI restriction fragment (503bp) including the DNA-binding domain of hRAR- $\alpha$ -encoding DNA (Giguere et al., Nature 330, 624 (1987); commonly assigned United States Patent Application Serial No. 276,536, filed November 30, 1988; European Patent Application Publication No. 0 325 849, all incorporated herein by reference) was nick-translated and used to probe a Southern blot of EcoRI-digested, genomic DNA of *Drosophila melanogaster* to identify potential homologs of vertebrate steroid hormone receptors. Under conditions of reduced hybridization stringency, six distinct EcoRI bands, ranging in size from 2 kilobases ("kb") to 12 kb, were detected (see Oro et al., Nature 336, 493 (1988)). Using the same probe and reduced stringency conditions, screening of a *D. melanogaster* genomic library in lambda-gt11 resulted in the isolation of three classes of inserts, based on cross-hybridization under high stringency conditions. Representatives of each class were hybridized to larval salivary gland polytene chromosomes to identify their cytogenetic location. One class of inserts mapped to 2C9 on the *D. melanogaster* first chromosome and was labelled XR2C. A portion of the XR2C genomic insert hybridizing most strongly to the

fragment of the hRAR-alpha-encoding DNA probe was designated pHX3.5, subcloned and sequenced. The deduced amino acid sequence of one of the reading frames in pHX3.5 had the structural features of a steroid receptor DNA binding domain.

pHX3.5 was used as a probe to screen a total third instar larval imaginal disc cDNA library from *D. melaogaster* in the EcoRI site of BluescriptR phagemid SK(+) (Stratagene, La Jolla, California, USA). Six cDNA clones were identified by this procedure. The complete nucleotide sequence of the longest, designated pXR2C8, except for the overhanging EcoRI ends, is shown in Figure 1, along with the deduced amino acid sequence of the 513 amino-acid XR2C encoded by the segment of pXR2C8 from nucleotides 163 - 1701.

The gene for XR2C has been mapped to the ultraspiracle locus of *D. melanogaster*, indicating that function of the XR2C receptor is essential for normal development and that interruption of the function early in development would be lethal.

#### Example II

Amino acid sequences of XR2C, hRXR-alpha (commonly owned United States Patent Application Serial No. 478,071, filed February 9, 1990, incorporated herein by reference), hRAR-alpha (human retinoic acid receptor-alpha) (Giguere et al., Nature 330, 624 (1987); commonly assigned United States Patent Application Serial No. 276,536, filed November 30, 1988; European Patent Application Publication No. 0 325 849, all incorporated herein by reference); and hGR (human glucocorticoid receptor) (Hollenberg et al., Nature 318, 635 (1985), commonly assigned United States Patent Application Serial No. 07/108,471, filed October 20, 1987, PCT International Publication No. WO 88/03168, all incorporated herein by reference), were aligned using the University of



Wisconsin Genetics Computer Group program "Bestfit" (Devereux et al., supra). Regions of significant similarity between XR2C and the other receptors, i.e., the 66 - 68 amino acid DNA binding domains and the  
5 ligand-binding domains) are presented schematically in Figures 2 as percent amino acid identity.

From Figure 2, it is clear that XR2C is more closely related to human RXR-alpha than to the other two receptors.

10 The DNA binding domain of XR2C is 66 amino acids in length (amino acids 104 - 169 of XR2C) and includes 10 cysteines.

#### Example III

15 *Drosophila melanogaster* Schneider line 2 ("S2") cells (Schneider, Embryol. Exp. Morphol. 27, 353 (1972), which are widely available and readily available to the skilled, are seeded at  $2 \times 10^6$  per 35 mm<sup>2</sup> culture dish and maintained in Schneider medium (GIBCO/Life Technologies,  
20 Inc., Grand Island, New York, USA) supplemented with penicillin, streptomycin and 12% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, California, USA). The cells are transiently cotransfected with 10 µg/dish plasmid DNA by calcium phosphate precipitation  
25 (Krasnow et al., Cell 57, 1031 (1989): 4.5 µg/dish effector construct or control construct (producing no XR2C); 0.5 µg/dish reporter plasmid or control reporter plasmid; 0.5 µg/dish reference plasmid; and 4.5 µg inert plasmid DNA.

30 In the effector construct, i.e., receptor expression vector (4.5 µg/dish), XR2C is constitutively expressed in the S2 cells under the control of the *Drosophila* actin 5C promoter (Thummel et al., Gene 74, 445 (1988)) driving transcription of the EcoRI-site-  
35 bounded insert of pXR2C8. In the control vector, (als 4.5

µg/ml), the EcoRI site bounded insert from plasmid is inserted in the reverse orientation (i.e., non-coding or non-sense-coding) orientation in comparison with the orientation in the effector construct. The effector construct is made by first inserting at the unique BamHI site of A5C a linker of sequence 5'-GATCCGATATCCATATGGAATTCGGTACCA and then inserting, in the XR2C-coding orientation, into the modified A5C at the EcoRI site of the linker the EcoRI-site-bounded insert of pXR2C8.

The reporter plasmid, i.e. a test vector, ADH-TRE<sub>p</sub>-CAT (at 0.5 µg/dish) contains the palindromic thyroid hormone response element TRE<sub>p</sub>, of sequence 5'-AGGTCATGACCT (Glass et al. Cell 54, 313 (1988); Thompson and Evans, Proc. Natl. Acad. Sci. (USA) 86, 3494 (1989), inserted into position -33 (with respect to the transcription start site) of a pD33-ADH-CAT background (Krasnow et al., Cell 57, 1031 (1989)). pD33-ADH-CAT is a plasmid with the distal promoter of the Drosophila melanogaster alcohol dehydrogenase gene linked operably for transcription to the bacterial (E. coli) chloramphenicol acetyltransferase ("CAT") gene, a gene for the indicator protein CAT. ADH-TRE<sub>p</sub>-CAT was made by inserting the oligonucleotide of sequence

25

5'-CTAGAGGTCATGACCT  
TCCAGTACTGGAGATC-5'

into the XbaI site at the aforementioned position -33 in pD33-ADH-CAT. pD33-ADH-CAT, without TRE<sub>p</sub>, served as a control reporter plasmid.

A reference plasmid containing the beta-galactosidase transcription unit driven by the actin 5C promoter also is transfected (0.5 µg/dish) along with pGEM DNA (4.5 µg/dish) (Promega, Madison, Wisconsin) to make up the final DNA concentration to 10 µg/dish. The

reference plasmid is made by inserting a BamHI-site bounded, beta-galactosidase-encoding segment into the unique BamHI site of A5C. The purpose of the reference plasmid was to normalize results for transfection efficiency.

24 h post-transfection, retinoic acid is added to the cultures. The retinoic acid is dissolved in ethanol and the resulting solution is added to 0.1 % v/v of culture medium. Initial concentration of the retinoic acid in the culture media is  $10^{-6}$  M,  $5 \times 10^{-7}$  M or  $10^{-8}$  M.

In control runs, ethanol, at 0.1 % v/v in the medium, is used in place of a solution of retinoid.

Cultures are maintained in the dark for 36 hr after addition of retinoic acid and then harvested. All other parts of the experiments were carried out in subdued light.

Cell lysates are centrifuged. Supernatants are assayed for beta-galactosidase, following Herbolme et al., Cell 39, 653-662 (1984), and units/ml of beta-galactosidase activity is calculated. CAT assays (normalized to beta-gal activity) of supernatants are incubated for 75 unit-hours ("units" referring to units of beta-gal activity), as described by Gorman et al., Mol. Cell. Biol. 2, 1044 (1982), usually 150 units for 30 minutes.

No XR2C-dependent activation of CAT expression is noted in any experiment in which control reporter is used in place of ADH-TREp-CAT. Similarly, essentially no activation is observed for runs where control effector plasmid is used in place of effector plasmid.

Results are expressed in terms of fold-induction of CAT activity in retinoic acid-treated cells in comparison with untreated (i.e., only ethanol-treated) cells.

The level of expression of CAT is observed to increase between the test cells exposed to an initial

concentration of retinoic acid of  $10^{-6}$  M (higher level of expression) and those exposed to an initial concentration of retinoic acid of  $5 \times 10^{-7}$  M. Similarly, the level of expression with test cells exposed to an initial  
5 concentra-tion of retinoic acid of  $5 \times 10^{-7}$  M is observed to be significantly higher than with cells exposed to an initial concentration of retinoic acid of  $10^{-8}$  M.

10

#### Example 4

To analyze insect DNA for homologs of XR2C, two genomic DNA Southern blots are prepared in parallel with identical DNA samples from insects of a particular species. The blots are hybridized at high or low  
15 stringency with a ~1300 bp [ $^{32}$ P]-labelled fragment of pXR2C8 which includes the coding portions of the DNA and ligand binding domains (nucleotides 472 - 1701, Fig.1 ) or an approx. 450 bp  $^{32}$ P-labelled PstI-BamHI fragment (approx. nucleotide 419 - approx. nucleotide 774)  
20 including the DNA encoding the DNA-binding domain (nucleotides 472 - 669, Fig. 1).

Blots are hybridized at 42 °C in a low stringency buffer (35 % formamide, 1 X Denhardt's, 5 X SSPE (1 X SSPE = 0.15 M NaCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM EDTA),  
25 0.1 % SDS, 10 % dextran sulfate, 100 mg/ml denatured salmon sperm DNA and 106 cpm of  $^{32}$ P-labelled probe) for low stringency hybridization or at high stringency in the same buffer modified by addition of formamide to 50 %.

Low stringency blots are washed twice at room temperature and twice at 50 °C in 2X SSC, 0.1% SDS. The high  
30 stringency blot is washed twice at room temperature in 2X SSC, 0.1% SDS and twice at 65° C in 0.5X SSC, 0.1% SDS.

#### Deposit

35

On November 10, 1989, viable cultures of E. coli DH5 transformed with pXR2C8 were deposited under the

terms of the Budapest Treaty on the International  
Recognition of the Deposit of Microorganisms for the  
Purposes of Patent Procedure at the American Type Culture  
Collection, Rockville, Maryland, USA ("ATCC"). The  
5 accession number assigned to this deposit is ATCC 68171.

Samples of E. coli DH5(pXR2C8) will be publicly  
available from the ATCC without restriction, except as  
provided in 37 CFR 1.801 et seq., at the latest on the  
date an United States Patent first issues on this  
10 application or a continuing application thereof.  
Otherwise, in accordance with the Budapest Treaty and the  
regulations promulgated thereunder, samples will be  
available from the ATCC to all persons legally entitled  
to receive them under the law and regulations of any  
15 country or international organization in which an  
application, claiming priority of this application, is  
filed or in which a patent based on any such application  
is granted.

Although the invention has been described  
20 herein with some specificity, those of skill in the art  
will recognize modifications and variations of what has  
been described that fall within the spirit of the  
invention. These modifications and variations are also  
intended to be within the scope of the invention as  
25 described and claimed.

## WHAT IS CLAIMED:

1. A substantially pure DNA sequence which encodes a polypeptide, wherein said polypeptide is  
5 characterized by:
  - (1) being responsive to the presence of retinoic acid to regulate the transcription of associated gene(s); and
  - (2) having a DNA binding domain of about 66  
10 amino acids with 10 Cys residues, wherein said DNA binding domain has:
    - (a) more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha, and  
15 (b) less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR.
2. A DNA according to Claim 1 wherein the  
20 polypeptide encoded by said DNA comprises a DNA binding domain with substantially the same sequence as that of amino acids 104 - 169 shown in Figure 1.
3. A DNA according to Claim 2 wherein the  
25 polypeptide encoded by said DNA has substantially the same sequence as that of amino acids 1 - 513 shown in Figure 1.
4. A DNA according to Claim 3 wherein said DNA  
30 comprises a segment with substantially the same nucleotide sequence as nucleotides 163 - 1704 shown in Figure 1.
5. A DNA according to Claim 4 which is pXR2C8.  
35

6. A DNA according to any one of Claims 1 - 4, wherein said DNA is contained in an expression vector which is operative in an insect cell in culture to make said polypeptide by expression of said DNA in said cell.

5

7. A DNA according to Claim 6 wherein transcription of said DNA is controlled by the *Drosophila melanogaster* actin 5C promoter.

10

8. An insect cell which is transformed with an expression vector according to Claim 6 or 7.

9. A cell according to Claim 8 which is a *Drosophila melanogaster* Schneider line 2 cell.

15

10. A cell according to Claim 8 or 9, wherein said cell is further transformed with a reporter vector which comprises:

20

- (a) a promoter that is operable in said cell,
- (b) a hormone response element, and
- (c) a DNA segment encoding a reporter protein, wherein said reporter protein-encoding DNA segment is operatively linked to said promoter for transcription of said DNA segment, and

25

wherein said hormone response element is operatively linked to said promoter for activation thereof.

30

11. A cell according to Claim 10 wherein: the promoter driving transcription of the reporter gene is the distal promoter of the *Drosophila melanogaster* alcohol dehydrogenase gene,

35

the hormone response element is selected from TREp or beta-RARE, and

the reporter protein is chloramphenicol acetyltransferase.

12. A cell according to Claim 11 wherein the  
5 reporter vector is the plasmid ADH-TREp-CAT.

13. A method of testing compound(s) for the  
ability thereof to activate the transcription-activating  
effects of receptor polypeptide(s), said method  
10 comprising:

assaying for the presence or absence of  
reporter protein upon contacting of cells containing  
receptor polypeptide and reporter vector with said  
compound(s);

15 wherein said receptor polypeptide is  
characterized by:

- (1) being responsive to the presence of  
retinoic acid to regulate the  
transcription of associated gene(s); and
- 20 (2) having a DNA binding domain of about 66  
amino acids with 10 Cys residues, wherein  
said DNA binding domain has:
  - (a) more than about 75 % amino acid  
identity in comparison with the DNA  
25 binding domain of hRXR-alpha, and
  - (b) less than about 60 % amino acid  
identity in comparison with the DNA  
binding domain of hGR, and

wherein said reporter vector comprises:  
30 (a) a promoter that is operable in said  
cell,  
(b) a hormone response element, and  
(c) a DNA segment encoding a reporter  
prot in,



wherein said receptor protein-encoding DNA segment is operatively linked to said promoter for transcription of said DNA segment, and

5 wherein said hormone response element is operatively linked to said promoter for activation thereof.

14. A method according to Claim 13 wherein said receptor polypeptide is produced by an expression  
10 vector, which is operative in the cells to make said polypeptide.

15 15. A method according to Claim 13 wherein the cells employed are *Drosophila melanogaster* Schneider line 2 cells co-transformed with:

(I) a DNA sequence which encodes a polypeptide, contained in an expression vector which is operative in an insect cell in culture to make  
20 said polypeptide by expression of said DNA in said cell, wherein said polypeptide is characterized by:

(1) being responsive to the presence of retinoic acid to regulate the  
25 transcription of associated gene(s); and

(2) having a DNA binding domain of about 66 amino acids with 10 Cys residues, wherein said DNA binding domain has:

30 (a) more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha, and

35 (b) less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR; and

(II) ADH-TREp-CAT.

16. A DNA or RNA which is labelled for detection and comprises a segment of at least 20 bases in length which has substantially the same sequence as either:

- 5 (i) a segment of the same length from the DNA segment from bases 1 - 2271, inclusive, of the DNA illustrated in Figure 1, or
- (ii) the complement of said segment.

10

17. A method of making a receptor polypeptide, wherein said receptor polypeptide is characterized by:

- (1) being responsive to the presence of retinoic acid to regulate the transcription of associated gene(s);
- 15 and
- (2) having a DNA binding domain of about 66 amino acids with 10 Cys residues, wherein said DNA binding domain has:
- 20 (a) more than about 75 % amino acid identity in comparison with the DNA binding domain of hRXR-alpha, and
- (b) less than about 60 % amino acid identity in comparison with the DNA binding domain of hGR,
- 25

said method comprising:

culturing cells which are transformed with an expression vector operable in said cells to express a DNA

30 which encodes said polypeptide.

18. A method according to Claim 17 wherein the protein encoded by said DNA comprises a DNA binding domain with substantially the same sequence as that of

35 amino acids 104 - 169 shown in Figure 1.

25

19. A method according to Claim 18 wherein the protein encoded by said DNA has substantially the same sequence as that of amino acids 1 - 513 shown in Figure 1.

5

20. A method according to Claim 20 wherein the DNA from which said protein is expressed comprises a segment with a nucleotide sequence substantially the same as the sequence of nucleotides 163 - 1704 shown in Figure 1.

10

## FIG. 1A

GGACACGGTG	GCGTTGGCAA	AGTGAACCC	CAACAGAGAG	GCGAAAGCGA	GCCAAGACAC	60
ACCACATACA	CACGAAGAGA	ACGAGCAAGA	AGAAACCGGT	AGGCGGAGGA	GCGGCTGCCC	120
CCAGTTCCTC	CAATATACCC	AGCACCACAT	CACAAGCCCA	GG ATG GAC AAC TGC		174
				Met Asp Asn Cys		
GAC CAG GAC GCC AGC TTT CGG CTG AGC CAC ATC AAG GAG GAG GTC AAG						222
Asp Gln Asp Ala Ser Phe Arg Leu Ser His Ile Lys Glu Glu Val Lys						
5 10 15 20						
CCG GAC ATC TCG CAG CTG AAC GAC AGC AAC AAC AGC AGC TTT TCG CCC						270
Pro Asp Ile Ser Gln Leu Asn Asp Ser Asn Asn Ser Ser Phe Ser Pro						
25 30 35						
AAG GCC GAG AGT CCC GTG CCC TTC ATG CAG GCC ATG TCC ATG GTC CAC						318
Lys Ala Glu Ser Pro Val Pro Phe Met Gln Ala Met Ser Met Val His						
40 45 50						
GTG CTG CCC GGC TCC AAC TCC GCC AGC TCC AAC AAC AGC AGC GCT GGA						366
Val Leu Pro Gly Ser Asn Ser Ala Ser Ser Asn Asn Ser Ala Gly						
55 60 65						
GAT GCC CAA ATG GCG CAG GCG CCC AAT TCG GCT GGA GGC TCT GCC GCC						414
Asp Ala Gln Met Ala Gln Ala Pro Asn Ser Ala Gly Gly Ser Ala Ala						
70 75 80						

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GCT GCA GTC CAG CAG CAG TAT CCG CCT AAC CAT CCG CTG AGC GGC AGC	462
Ala Ala Val Gln Gln Gln Tyr Pro Pro Asn His Pro Leu Ser Gly Ser	100
85	90
95	
AAG CAC CTC TGC TCT ATT TGC GGC GAT CGG GCC AGT GGC AAG CAC TAC	510
LYS His Leu Cys Ser Ile Cys Gly Asp Arg Ala Ser Gly Lys His Tyr	115
105	110
GGC GTG TAC AGC TGT GAG GGC TGC AAG GGC TTC TTT AAA CGC ACA GTG	558
Gly Val Tyr Ser Cys Glu Gly Cys Lys Gly Phe Phe Lys Arg Thr Val	130
120	125
CGC AAG GAT CTC ACA TAC GCT TGC AGG GAG AAC CGC AAC TGC ATC ATA	606
Arg Lys Asp Leu Thr Tyr Ala Cys Arg Glu Asn Arg Asn Cys Ile Ile	145
135	140
GAC AAG CGG CAG AGG AAC CGC TGC CAG TAC TGC CGC TAC CAG AAG TGC	654
ASP Lys Arg Gln Arg Arg Asn Arg Cys Gln Tyr Cys Arg Tyr Gln Lys Cys	160
150	155
CTA ACC TGC GGC ATG AAG CGC GAA GCG GTC CAG GAG GAG CGT CAA CGC	702
Leu Thr Cys Gly Met Lys Arg Glu Ala Val Gln Glu Glu Arg Gln Arg	180
165	170
GGC GCC CGC AAT GCG GCG GGT AGG CTC AGC GCC AGC GGA GGC GGC AGT	750
Gly Ala Arg Asn Ala Ala Gly Arg Leu Ser Ala Ser Gly Gly Ser	195
185	190

FIG. 1B

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AGC GGT CCA GGT TCG GTA GGC GGA TCC AGC TCT CAA GGC GGA GGA GGA	798
Ser Gly Pro Gly Ser Val Gly Gly Ser Ser Ser Ser Gly Gly Gly Gly	200 205 210
GGA GGC GGC GTT TCT GGC GGA ATG GGC AGC AAC GGT TCT GAT GAC	846
Gly Gly Gly Val Ser Gly Gly Met Gly Ser Gly Asn Gly Ser Asp Asp	215 220 225
TTC ATG ACC AAT AGC GTG TCC AGG GAT TTC TCG ATC GAG CGC ATC ATA	894
Phe Met Thr Asn Ser Val Ser Arg Asp Phe Ser Ile Glu Arg Ile Ile	230 235 240
GAG GCC GAG CAG CGA GCG GAG ACC CAA TGC GGC GAT CGT GCA CTG ACG	942
Glu Ala Glu Gln Arg Ala Glu Thr Gln Cys Gly Asp Arg Ala Leu Thr	245 250 255 260
TTC CTG CGC GTT GGT CCC TAT TCC ACA GTC CAG CCG GAC TAC AAG GGT	990
Phe Leu Arg Val Gly Pro Tyr Ser Thr Val Gln Pro Asp Tyr Lys Gly	265 270 275
GCC GTG TCG GCC CTG TGC CAA GTG GTC AAC AAA CAG CTC TTC CAG ATG	1038
Ala Val Ser Ala Leu Cys Gln Val Val Asn Lys Gln Leu Phe Gln Met	280 285 290
GTC GAA TAC GCG CGC ATG ATG CCG CAC TTT GCC CAG GTG CCG CTG GAC	1086
Val Glu Tyr Ala Arg Met Met Pro His Phe Ala Gln Val Pro Leu Asp	295 300 305
GAC CAG GTG ATT CTG CTG AAA GCC GCT TGG ATC GAG CTG CTC ATT GCG	1134
Asp Gln Val Ile Leu Leu Lys Ala Ala Trp Ile Glu Leu Leu Ile Ala	310 315 320

FIG. 1C

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AAC GTG GCC TGG TGC AGC ATC GTT TCG CTG GAT GAC GGC GGT GCC GGC	1182
Asn Val Ala Trp Cys Ser Ile Val Ser Leu Asp Asp Gly Gly Ala Gly	325 330 335 340
GGC GGC GGT GGA CTA GGC CAC GAT GGC TCC TTT GAG CGA CGA TCA	1230
Gly Gly Gly Gly Leu Gly His Asp Gly Ser Phe Glu Arg Arg Ser	345 350 355
CCG GGC CTT CAG CCC CAG CAG CTG TTC CTC AAC CAG AGC TTC TCG TAC	1278
Pro Gly Leu Gln Pro Gln Gln Leu Phe Leu Asn Gln Ser Phe Ser Tyr	360 365 370
CAT CGC AAC AGT GCG ATC AAA GCC GGT GTG TCA GCC ATC TTC GAC CGC	1326
His Arg Asn Ser Ala Ile Lys Ala Gly Val Ser Ala Ile Phe Asp Arg	375 380 385
ATA TTG TCG GAG CTG AGT GTA AAG ATG AAG CCG CTG AAT CTC GAC CGA	1374
Ile Leu Ser Glu Leu Ser Val Lys Met Lys Arg Leu Asn Leu Asp Arg	390 395 400
CGC GAG CTG TCC TGC TGG AAG GCC ATC ATA CTG TAC AAC CCG GAC ATA	1422
Arg Glu Leu Ser Cys Leu Lys Ala Ile Ile Leu Tyr Asn Pro Asp Ile	405 410 415 420
CGC GGC ATC AAG AGC CCG GCG GAG ATC GAG ATG TGC CGC GAG AAG GTG	1470
Arg Gly Ile Lys Ser Arg Ala Glu Ile Glu Met Cys Arg Glu Lys Val	425 430 435

FIG. 1D

## FIG. 1E

TAC GCT TGC CTG GAC GAG CAC TGC CGC CTG GAA CAT CCG GGC GAC GAT 1518  
 Tyr Ala Cys Leu Asp Glu His Cys Arg Leu Glu His Pro Gly Asp Asp 450  
 440 445  
 GGA CGC TTT GCG CAA CTG CTG CTG CGT CTG CGC CGC TTT GCG ATC GAT 1566  
 Gly Arg Phe Ala Gln Leu Leu Leu Arg Arg Arg Phe Ala Ile Asp 460  
 455 465  
 CAG CCT GAA GTG CCA GGA TCA CCT GTT CCT CTT CCG CAT TAC CAG CGA 1614  
 Gln Pro Glu Val Pro Gly Ser Pro Val Pro Leu Pro His Tyr Gln Arg 475  
 470 480  
 CCG GCC GCT GGA GGA GCT CTT TCT CGA GCA GCT GGA GGC GCC GCC GCC 1662  
 Pro Ala Ala Gly Gly Ala Leu Ser Arg Ala Ala Gly Gly Ala Ala Ala 490  
 485 495 500  
 ACC CGG CCT GGC GAT GAA ACT GGA GTA GGG TCC CGA CTC T AAAGTCGCC 1712  
 Thr Arg Pro Gly Asp Glu Thr Gly Val Gly Ser Arg Leu 505  
 510

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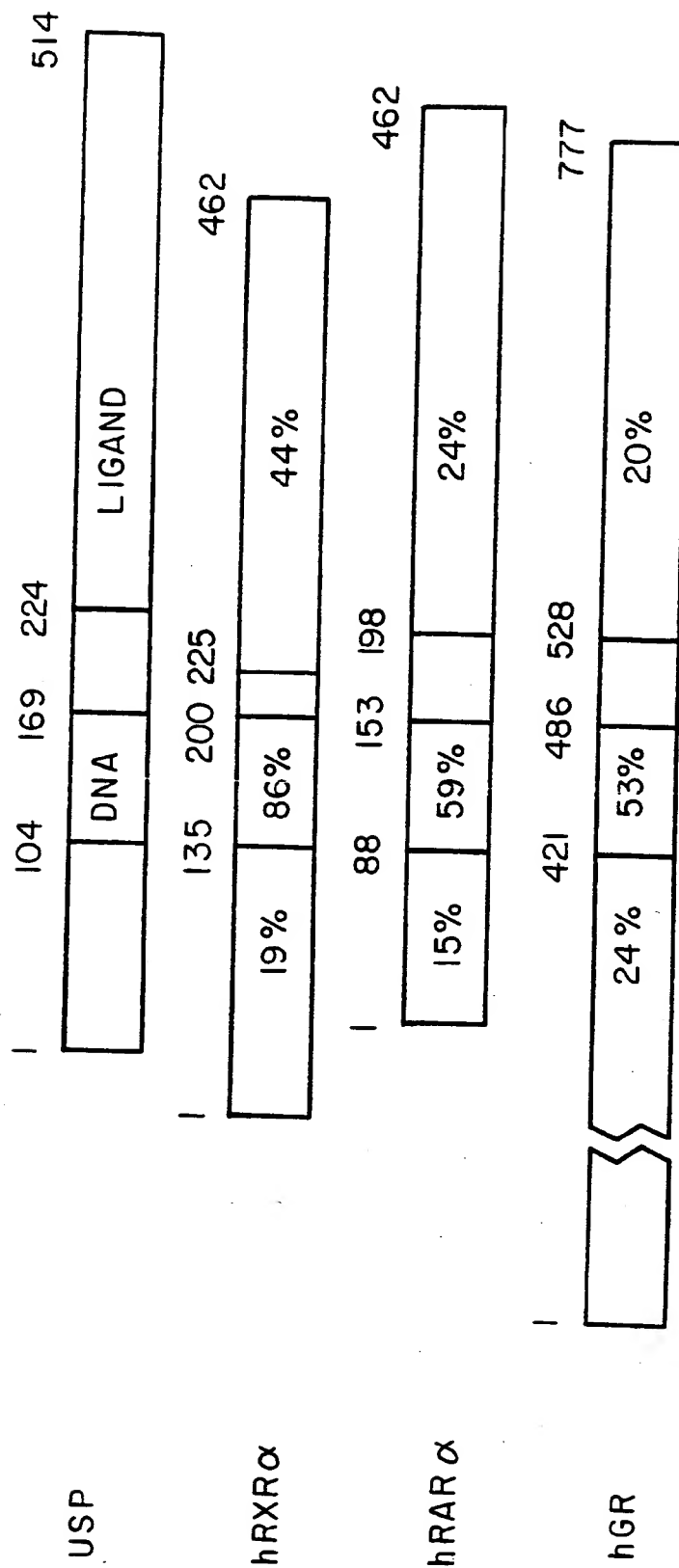
CCGTTCTCCA TCCGAAAAAT GTTTCATTGT GATTGCGTTT GTTGCATTT CTCCTCTCTA 1772  
TCCCTACAAA AGCCCCCTAA TATTACGCAA AATGTGTATG TAATTGTTA TTTTTTTTTT 1832  
ATTACCTAAT ATTATTATTA TTATTGATAT AGAAAATGTT TTCCTTAAGA TGAAGATTAG 1892  
CCTCCTCGAC GTTTATGTCC CAGTAAACGA AAAACAACA AAATCCAAAA CTGAAAAAGA 1952  
ACACAAAACA CGAACGAGAA AATGCACACA AGCAAAGTAA AAGTAAAGT TAAACTAAAG 2012  
CTAAACGAGT AAAGATATTA AAATAACGGT TAAAATTAAAT GCATAGTTAT GATCTACAGA 2072  
CGTATGTAAA CATACAAATT CAGCATAAAT ATATATGTCA GCAGGCGCAT ATCTGCGGTG 2132  
CTGGCCCCGT TCTAAACCAA TTGTAATTAC TTTTAAACAT AAATTACCC AAAACGTTAT 2192  
CAATTAGATG CGAGATACAA AAATCACCAG CGAAAACCAA CAAAATATAT CTATGTATAA 2252  
AAAATATAAG CTGCATAACA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AA 2304

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FIG. 1F

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FIG. 2



# INTERNATIONAL SEARCH REPORT

International Appl. No. PCT/US91/01894

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate them all)		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C07H 5/12; C12N 15/00, 5/00, 1/00 U.S. CL.: 536/27; 435/172.1; 435/320; 435/240.1; 435/243		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>1</sup>		
Classification System	Classification Symbols	
US	536/27; 435/172.1; 435/320; 435/240.1; 435/243	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>1</sup>		
APS and CAS data bases		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>2</sup></b>		
Category <sup>3</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
<u>X</u> Y	Nature, Volume 336, issued 01 December 1988, <b>ORO ET AL.</b> "The <u>Drosophila</u> gene <u>knirps-related</u> is a member of the steroid-receptor gene superfamily", pages 393-396; see abstract, figure 2.	<u>1</u> 2-12
A	EP, A, 0,325,849 ( <b>EVANS ET AL.</b> ) 02 August 1989.	1-12
<u>X</u> Y	Nucleic Acids Research, Volume 17, number 18, <b>FEIGL ET AL.</b> , "A member of the steroid hormone receptor gene family is expressed in the 20-OH-ecdysone inducible puff 75B in <u>Drosophila melanogaster</u> ", pages 7167-78, see abstract, figure 2.	<u>1</u> 2-12
A	WO 89/05355 ( <b>EVANS ET AL.</b> ) 15 June 1989.	1-12
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubt on priority claim(s) or which is cited to establish the priority date of a claim or other citation or other special reason (is specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to reside in the prior art</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is considered with the knowledge of such documents, such as contained in the prior art</p> <p>"Z" document mentioned in the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
19 June 1991	10 JUL 1991	
International Searching Authority	Inventor's Authorized Certificate	
ISA/US	Jerry Massie (vsh)	

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## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See attachment to telephone memorandum.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-12
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not make payment of any additional fee.

## Remarks on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

BNSDOCID: 2403090489541 PAST Gazette No. 17/1992, Section II)

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CI	Côte d'Ivoire	LI	Liechtenstein	SU <sup>+</sup>	Soviet Union
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CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE <sup>+</sup>	Germany	MC	Monaco	US	United States of America
DK	Denmark				

<sup>+</sup> Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

REVISED  
VERSION

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01894

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>  
According to International Patent Classification (IPC) or to both National Classification and IPC  
IPC(5): C07H 15/12; C12N 15/00, 5/00, 1/00  
U.S. CL.: 536/27; 435/172.1; 435/320; 435/240.1; 435/243

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System

Classification Symbols

US

536/27; 435/172.1; 435/320; 435/240.1; 435/243

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

APS and CAS data bases

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
<u>X</u> Y	Nature, Volume 336, issued 01 December 1988, <b>ORO ET AL.</b> "The <u>Drosophila</u> gene <u>knirps-related</u> is a member of the steroid-receptor gene superfamily", pages 393-396; see abstract, figure 2.	<u>1</u> 2-12
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

19 June 1991

Date of Making of this International Search Report

10 JUL 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

*Jerry Massie*  
for Jerry Massie

(vsh)

V ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

- 1 ☐ Claim numbers \_\_\_\_\_ because they relate to subject matter not required to be searched by this Authority, namely:
- 2 ☐ Claim numbers \_\_\_\_\_ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
- 3 ☐ Claim numbers \_\_\_\_\_ because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

- 1 ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
  - 2 ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  - 3 ☒ As required additional search fees were timely paid by the applicant, consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-12
  - 4 ☐ As all searchable claims could be searched without effecting an additional fee, the International Searching Authority is not obliged to restrict the scope of the invention.
- Objection to Protest
- ☐ The international search fees were accompanied by applicant's protest.
  - ☐ The protest accompanied the payment of additional search fees.

